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Apoptosis Induction Mediated by Wild-Type *p53* Adenovirus Gene Transfer in Squamous Cell Carcinoma of the Head and Neck[1]

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ABSTRACT

Cancer gene therapy strategies for inducing apoptosis in solid tumors may allow contemporary medicine to reassess its management of these cancers. We demonstrated previously that overexpression of the wild-type *p53* gene in squamous cell carcinoma of the head and neck cell lines via adenovirus-mediated gene transfer suppressed growth both *in vitro* and *in vivo*. Here, we characterize the mechanism of the growth suppression by the exogenous *p53* gene as a consequence of programmed cell death (apoptosis). One of the cell lines used in this study, Tu-138, harbors a mutated *p53* gene, whereas the other cell line, MDA 686LN, possesses a wild-type *p53* gene. DNA fragmentation was detected by electrophoresis in both cell lines after infection with the wild-type *p53* adenovirus, Ad5CMV-*p53*. With the use of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling method, 4.4% of the remaining viable Tu-138 cell population was identified as apoptotic as early as 15 h after inoculation with Ad5CMV-*p53*. The percentage of apoptotic cells increased to 31% at 22 h. In contrast, only 10% of the viable MDA 686LN cells (wt-*p53*) had undergone apoptosis 30 h after Ad5CMV-*p53* infection, although the percentage of apoptotic cells rapidly increased to 60% at 48 h after infection. For *in vivo* analysis of apoptosis, nude mice in which squamous cell carcinoma of the head and neck cell lines had been implanted s.c. had exogenous wt-*p53* transiently introduced to the tumor cells via Ad5CMV-*p53* 2 days later. *In situ* end labeling clearly illustrated apoptosis in the tumor cells. These results suggest that wt-*p53* plays an important role in the induction of apoptosis in human head and neck cancer cell lines and that selective induction of apoptosis in cancer cells can be further explored as a strategy for cancer gene therapy.

INTRODUCTION

Balancing the rates of cell proliferation and cell death is important in maintaining normal tissue homeostasis. Disruption of this balance may be a major factor in the multistep process of tumorigenesis, and inhibition of apoptosis, or programmed cell death, is one cause of this disruption. Apoptosis also occurs during normal embryogenesis, in the course of normal tissue turnover, after withdrawal of a trophic hormone from its target tissue, and in thymic regression, offering excellent opportunities to study the apoptotic process. We contend that the most promising new therapies for solid malignancies are interventions at the molecular level, and that selective induction of apoptosis in these cancers is a logical intervention strategy. The gene or genes that may induce cancer cell apoptosis continue to be investigated, as do the methods for gene transfer. Presently, adenovirus-mediated gene transfer is our clinical method of choice for such interventions because of its known tropism for the epithelium of the aerodigestive tract, its excellent transduction efficiency, the transient nature of gene expression in the cells it infects (lack of permanent integration), and its ability to infect nonproliferating cells. The *p53* gene also continues to be of interest as a molecular therapy for some solid malignancies.

p53 was originally discovered through its association with the SV40 large T antigen (1, 2). The importance of the *p53* gene product in human neoplasia was first recognized a few years ago when mutant forms of the gene were identified in human colorectal tumors. Subsequently, *p53* mutations have been identified in the majority of human malignant solid tumors, including those of the breast (3), colon (4), lung (5), and oral cavity (6). Several studies have demonstrated the ability of the wt-*p53*[3] to suppress cancer cell growth both *in vitro* and *in vivo*, suggesting that it acts as a tumor suppressor gene. The suppression of cell growth by *p53* is mediated by two distinct pathways, one transient and one permanent. In the case of transient suppression, *p53* serves as a cell cycle checkpoint regulator. Overexpression of wt-*p53* has been shown to induce a reversible cell cycle arrest at the G_1-S boundary (7, 8). In other instances, *p53* may induce apoptosis when overexpressed in some cultured cells (9, 10) and is required for DNA damage-induced apoptosis in mouse thymocytes (11).

We reported previously that overexpression of the wt-*p53* in SCCHN cell lines induced via adenovirus-mediated gene transfer suppressed growth both *in vitro* and *in vivo* (12, 13). Here, we sought to examine the mechanism of this growth suppression. Our data indicate that the suppression effect, both *in vitro* and *in vivo*, is the consequence of an irreversible event, apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions: Recombinant Adenovirus Preparation and Infection. Human SCCHN cell lines Tu-138 and MDA 686LN were all established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center, and had been characterized previously (12, 13). All procedures were performed and cell lines maintained as described previously (12, 13). Cell growth assays were performed in triplicate.

DNA Fragmentation Analysis. After incubation with Ad5CMV-*p53* or replication-defective adenoviral controls at various time intervals, cells were harvested and resuspended in 300 μl of PBS, to which 3 ml of extraction buffer [10 mM Tris (pH 8.0)-0.1 M EDTA-20 μg/ml RNAse-0.5% SDS] were added before incubation at 37°C for 1–2 h. At the end of incubation, proteinase K was added to a final concentration of 100 μg/ml, and the solution was placed in a 50°C water bath for at least 3 h. DNA was extracted once with an equal volume of 0.5 M Tris (pH 8.0)-saturated phenol and then again with phenol/chloroform. Precipitated DNA was analyzed in a 1% agarose gel.

Cell Fixation. Before the TUNEL method was used to identify apoptotic cells, the cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 30 min on ice. Cells were then washed with 3 ml of PBS, resuspended in 70% ice-cold ethanol, and stored at −20°C until used. For cell cycle analysis, cells were fixed in 70% ice-cold ethanol only.

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[3] The abbreviations used are: wt-*p53*, wild-type *p53*; SCCHN, squamous cell carcinoma of the head and neck; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; Ad5CMV-*p53*, wild-type *p53* adenovirus; *Tdt*, terminal deoxynucleotidyl transferase.

Terminal Deoxynucleotidyl Transferase Assay. The TUNEL assay was performed according to the procedure of Gorczyca *et al.* (14). Briefly, after fixation and washing, cells were resuspended in 50 μl of TdT buffer containing 0.2 M sodium cacodylate (pH 7.0), 2.5 mM Tris-HCl, 2.5 mM $CoCl_2$ (Sigma Chemical Co., St. Louis, MO), 0.1 mM DTT (Sigma Chemical Co.), 0.25 mg/ml bovine serum albumin (Sigma Chemical Co.), 5 units of terminal transferase (Boehringer Mannheim, Indianapolis, IN), and 0.5 nmol biotin-16-dUTP along with dATP, dGTP, and dCTP at concentrations of 20 μM. Controls were prepared by incubating a separate aliquot of each test sample without dUTP. The cells were incubated in the solution at 37°C for 30 min, rinsed in PBS, and resuspended in 100 μl of fluorescein isothiocyanate, the staining solution containing 4X SSC, 0.1% Triton X-100, and 2.5 μg/ml fluoresceinated avidin (Vector Laboratories, Inc., Burlingame, CA). Tubes were incubated for 30 min in the dark at room temperature. Cells were rinsed in PBS with 0.1% Triton X-100 and resuspended in 0.5 ml PBS containing propidium iodide (5 μg/ml) and 70 μl (1 mg/ml) RNAse. Tubes were incubated in the dark on ice for 30 min before flow cytometric analysis.

Flow Cytometric Analysis. All samples were analyzed with the use of an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL) with the standard optical configuration. At least 10,000 events were counted for each sample. Positivity for TdT end labeling was determined by subtracting the control histogram from the test histogram with the use of the Immuno-4 program of the Elite workstation software (Coulter Corp.).

Cell Growth Assay. Cells were plated at a density of 2×10^4 cells/ml in 6-well plates in triplicate. Cells were infected with either the Ad5CMV-*p53* or the replication-defective adenovirus (dl312) as a control. Cells were harvested at different time intervals and counted, and their viability was determined by trypan blue exclusion.

***In Vivo* Analysis for Apoptosis.** Gene therapy in a microscopic residual disease model of SCCHN has been described elsewhere (13). Experiments were reviewed and approved by the institutional committees for both animal care and utilization and the Biosafety Committee for recombinant DNA research. Briefly, s.c. flaps were elevated in anesthetized 4–6-week-old nude female mice with sharp dissection, and 2.5×10^6 tumor cells in 100 μl of culture medium were pipetted into the flap and sealed with a horizontal mattress suture. Forty-eight h after tumor cell delivery, animals were reanesthetized and infected with Ad5CMV-*p53*, replication-defective virus (dl312), or PBS alone (mock infection). The animals were observed daily and killed 72 h after the second, or "therapeutic," intervention.

***In Situ* End Labeling.** The procedure was performed as described elsewhere (15). Briefly, paraffin-embedded tumor sections were dewaxed in xylene for 5 min three times each and were progressively hydrated by immersing the slides for 3 min each in 100, 90, 70, and 30% ethanol solutions. Endogenous peroxidase was inactivated by immersing the slides for 20 min in 0.75% H_2O_2 (v/v) in 100% methanol. After the slides were washed in PBS, sections were digested with 0.1% pepsin (Fisher Scientific, Houston, TX; w/v) in 0.1 N HCl for 5 min at 37°C and extensively washed in PBS. Sections were then incubated in a moist chamber at 37°C for 1 h with an end-labeling cocktail that included the following: 0.5 unit/μl TdT; 0.06 mM biotinylated dUTP; 10 μl 5× TdT buffer; and double-distilled water up to 50 μl. The reaction was terminated by immersing the slides in a buffer containing 300 mM NaCl and 30 mM sodium citrate in double-distilled water. After the slides were washed in PBS, sections were incubated with horseradish peroxide-conjugated avidin for 1 h at 37°C in a moist chamber. Staining was developed using 3,3'-diaminobenzidine, and sections were counterstained with methyl green.

RESULTS

Suppression of SCCHN Cell Line Growth by Ad5CMV-*p53*. We reported previously that wt-*p53* can be efficiently transduced into SCCHN cell lines by a recombinant adenoviral vector. Consequently, the insulted tumor cells lose their ability to proliferate *in vitro* as well as *in vivo*. The suppression effect is independent of the endogenous *p53* status of the cell lines. Previous growth rate analyses were carried out through a 1-week period. Here, we sought to investigate the early effects of the wt-*p53* on SCCHN cell growth (*i.e.*, after shorter time intervals).

Two representative cell lines were used in this study. Cell line Tu-138 harbors a mutated *p53* gene, whereas cell line MDA 686LN possesses a wt-*p53* gene. Cells infected with the replication-defective virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 1, *A* and *B*). On the other hand, growth of the Ad5CMV-*p53*-infected Tu-138 (Fig. 1*A*) and MDA 686LN (Fig. 1*B*) cells was significantly suppressed. It appeared that the exogenous p53 protein had an earlier and more profound suppressive effect on Tu-138 than on MDA 686LN. An apparent morphological change was observed in both cell lines, with portions of the cell populations rounding up and their outer membranes forming blebs. This resembled apoptosis and occurred concomitantly with the initiation of the growth suppression. Cells infected with the replication-defective adenovirus dl312 demonstrated normal growth characteristics with no histomorphological abnormalities. It is important that these effects were not observed after infection by Ad5CMV-*p53* of karyotypically normal fibroblasts (13) or human oral keratinocytes (immortalized but nontumorigenic with endogenous wt-*p53*).[4]

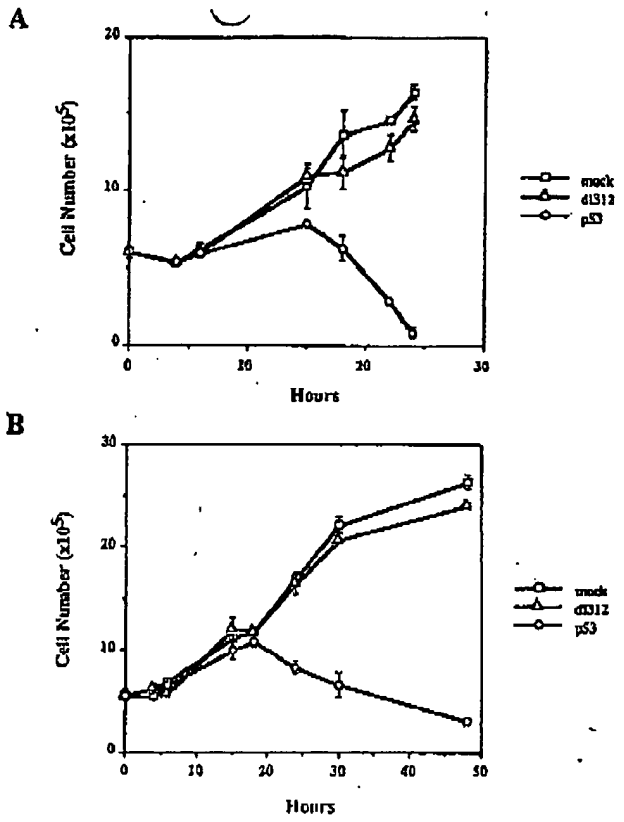

Fig. 1. Composite growth curve of SCCHN cell lines. *A*, Tu-138; *B*, MDA 686LN. At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate dishes were plotted against the number of hours postinfection. *Bars*, SEM.

DNA Fragmentation Analysis. One of the characteristic markers of apoptosis that distinguishes it from necrosis is the biochemically observable appearance of the ladder of DNA fragments. To confirm the assumption that the cells had undergone apoptosis after the Ad5CMV-*p53* infection, we performed DNA fragmentation analysis. Chromosomal DNA extracted from the viable cells after infection with the replication-defective virus or Ad5CMV-*p53* was subjected to agarose gel electrophoresis. The appearance of DNA fragments equivalent to approximately 200 bp, and their multiples

[4] G. L. Clayman, unpublished data.

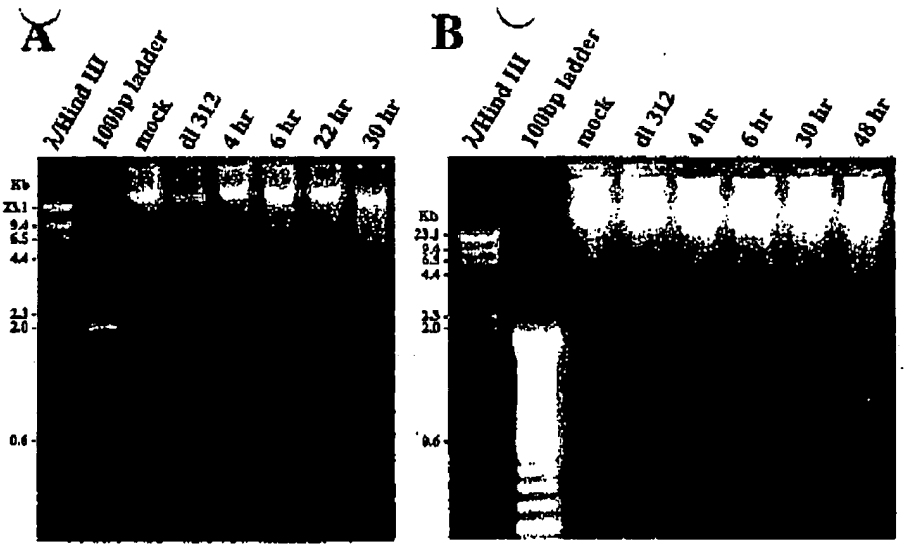

Fig. 2. *In vitro* DNA fragmentation analysis after infection with Ad5CMV-*p53*, mock virus, or replication-defective virus: *A*, Tu-138 cells (endogenous mutated *p53*); *B*, MDA 686LN cells (endogenous wt-*p53*). Both SCCHN cell lines were analyzed in a time course experiment. *Lanes 1* and *2* (from *left*), λ/*Hind*III and 100-bp DNA ladders served as molecular markers, respectively; *Lane 3*, mock infection at 24 h; *Lane 4*, replication-defective virus infection at 24 h; *Lanes 5* and *6*, Ad5CMV-*p53* infection at 4 and 8 h, respectively; *Lanes 7* and *8*, Ad5CMV-*p53* infection at time periods as shown.

was noticed in both cell lines (Fig. 2). The fragmented DNA appeared 22 h after Ad5CMV-*p53* infection in the Tu-138 cell line (Fig. 2*A*) and at 30 h in the MDA 686LN cell line (Fig. 2*B*), although they were more evident in the latter at 48 h. No detectable fragmented DNA emerged from the mock-infected or dl312-infected cells.

***In Vitro* TdT Assay.** Another characteristic marker of apoptosis is the morphological change and destruction of the structural organization of the nucleus, which results in chromatin condensation. Electron microscopy has been used extensively to detect such ultrastructural alteration. Recently, however, flow cytometric methods for identifying apoptotic cells have gained favor because of their greater ability to scan and analyze cellular populations (14). The TUNEL method used here is based on detection of the extensive DNA breakage to identify the apoptotic cells. Fifteen h after infection with Ad5CMV-*p53*, 4.4% of the viable Tu-138 cell population was in apoptotic stages (Fig. 3*A*), yet we were unable to detect any fragmented DNA in their agarose gels done at that time.[4] There appeared to be a delay in apoptosis induction in the MDA 686LN cells, with 4% of cells exhibiting detectable apoptosis 18 h after the infection with Ad5CMV-*p53* (Fig. 3*B*). This apparent discrepancy between the TUNEL assay and fragmentation analysis is most likely due to the high sensitivity of the TUNEL assay. The number of apoptotic cells increased proportionally to the duration of the Ad5CMV-*p53* incubation. Nearly 31% of the Tu-138 cells had undergone apoptosis at 22 h. Although induction of apoptosis was at first delayed in MDA 686LN cells, approximately 60% of these cells were in apoptotic stages 48 h after Ad5CMV-*p53* infection. It is noteworthy that the percentage of apoptotic cells may have been significantly underestimated by the TUNEL method because only viable cells were subjected to the analysis. These data correlated well with the growth rate and DNA fragmentation analyses. There was no detectable apoptosis in control experiments with the use of mock infection or replication-defective viral controls (100 multiplicity of infection). Therefore, apoptosis did not appear to be a function of the transduced adenoviral gene products themselves. Subsequently, cells were counterstained with propidium iodide for their total DNA content and visualized with the use of nuclear fluorescence (Fig. 4, *A–D*). Fig. 4, *A* and *B* (Tu-138) and *C* and *D* (MDA 686LN), reveals that cells exhibit orange nuclear fluorescence with normal nuclear anatomy after infection with replication-defective virus (Fig. 4, *A* and *C*). In contrast, cells infected with Ad5CMV-*p53* showed green fluorescence with nuclear fragmentation (Fig. 4, *B* and *D*).

***In Vivo* Analysis for Apoptosis.** We showed previously that Ad5CMV-*p53* could suppress tumor formation *in vivo*. In this study, we investigated whether this suppression was the consequence of apoptosis. *In situ* end-labeling analysis was performed to detect apoptotic cells in paraffin-embedded sections obtained from our previous study (13, 15). No staining was observed in the tissue sections isolated from MDA 686LN-bearing animals which had received PBS treatment only as controls (Fig. 4*E*). On the other hand, tissue sections isolated from MDA 686LN-bearing mice treated with Ad5CMV-*p53* were highly stained (Fig. 4*F*), suggesting that apoptosis was the event involved in suppression of tumor growth *in vivo*.

It has been shown that the *WAF1/CIP1* gene, which may be induced after wt-*p53* overexpression, is involved in cell cycle arrest (16). We sought to determine whether the tumor growth suppression we saw was due in part to the cell cycle arrest by the induced p21 protein or primarily to apoptosis. Western blotting showed that the p21 protein was induced in the Ad5CMV-*p53*-infected SCCHN cells. However, cell cycle analyses indicated that despite the elevated level of p21 protein in the Ad5CMV-*p53*-infected cells, there was no significant accumulation of cells at G_1 as compared to S phase.[4]

DISCUSSION

Tumor suppressor genes are only one of several groups of genes the transfer of which might be useful for the local and regional treatment of solid malignancies. Certainly, because of considerable evidence implicating mutations of the *p53* gene in human cancers, this has been one of the most extensively studied tumor suppressor genes to date. Studies have demonstrated that the growth of several different human cancer cell lines including colon (9), breast (17), osteosarcoma (18), and non-small cell lung cancer (19), can be functionally suppressed by wt-*p53* with the use of a variety of methods of gene transfer. The mechanism through which wt-*p53* expression mediates its control, however, requires further elucidation.

wt-*p53* has been shown to be involved in several aspects of cell growth control (20, 21). One of the functions of wt-*p53* as a tumor suppressor gene is to induce apoptosis in damaged cells. This process, however, is dependent on several known but yet to be characterized factors, not to mention cell type of origin and induced downstream effectors (9, 10).

We demonstrated previously that the introduction of wt-*p53* into SCCHN cell lines by a recombinant adenoviral vector suppressed the

growth of tumors both *in vitro* and *in vivo*. In study presented here, we sought to determine the mechanism responsible for the irreversible cytocidal effect of the recombinant wt-*p53* adenovirus in SCCHN.

Because transfer and overexpression of wt-*p53* initiated tumor growth suppression, and the wt-p53 protein is one of several gene products that may induce apoptosis, we investigated whether apoptosis was the mechanism involved in SCCHN. *In vitro* growth analysis showed that the growth of both SCCHN cell lines was suppressed by the exogenous expression of wt-p53 protein, independent of their endogenous *p53* status (whether mutated or wt). The effect occurred earlier and more profoundly in the endogenously mutated cell line, but growth was totally suppressed within 72 h in both of the SCCHN cell lines used (13). Whether this delay in suppression was a function of the endogenous p53 status of that cell line or of other related or unrelated molecular events remains to be investigated. It is important that karyotypically normal human fibroblasts (with endogenous wt-*p53*) were insensitive to Ad5CMV-*p53* treatment despite adequate transduction. Similarly, nonmalignant human immortalized oral keratinocytes were equally unaffected by such treatment. These studies illustrate the specificity of this molecular approach, as well as its sparing of normal tissues, even if they are transduced.

The SCCHN cell lines infected with Ad5CMV-*p53* clearly manifested apoptosis. Nucleosomal DNA analysis revealed characteristic fragmented DNA laddering in both cell lines, with fragmentation occurring earlier in the endogenously mutated cells. Replication-defective virus produced no effect, suggesting that the process was not a function of transduced gene products unrelated to the *p53* gene. *In vitro* TdT analysis corroborated both growth rate and DNA fragmentation analyses, although the assay proved to be more sensitive for the detection of earlier nuclear fragmentation and allowed the perusal of the overall tumor cell population. Moreover, *in situ* end labeling illustrated that apoptosis was also the mechanism involved in suppression of tumor growth *in vivo*.

Multiple pathways may exist for induction of apoptosis, and these pathways may be regulated by interventions from upstream and/or downstream factors. Accumulating evidence suggests that both *p53*-dependent and *p53*-independent apoptotic pathways exist (11, 22, 23). It is likely that multiple factors determine whether *p53* mediates cell cycle arrest or apoptosis, including the histological source and embryonic origin of the cells, the constitutive molecular make-up of the cells, and factors relating to the transformation process. *p53* is probably not a requirement for apoptosis in embryonic development because "*p53* knock-out" mice develop normally (24). In contrast, *p53* may be extremely important as a critical defense mechanism to induce apoptosis in cells that overexpress oncogenes or mutated tumor suppressor genes or are virally transformed. This has been shown in a Burkitt's lymphoma cell line which constitutively expressed an active *c-myc* oncogene and underwent apoptosis after expression of exogenous *p53* (25). Additionally, other factors, including the *Bcl* family of genes and adenoviral E1B proteins, may block apoptosis in both *p53*-dependent and *p53*-independent pathways (26).

The induction of *WAF1/CIP1* in *p53*-mediated G_1 arrest and apoptosis in response to DNA damage has also been reported (16). In our

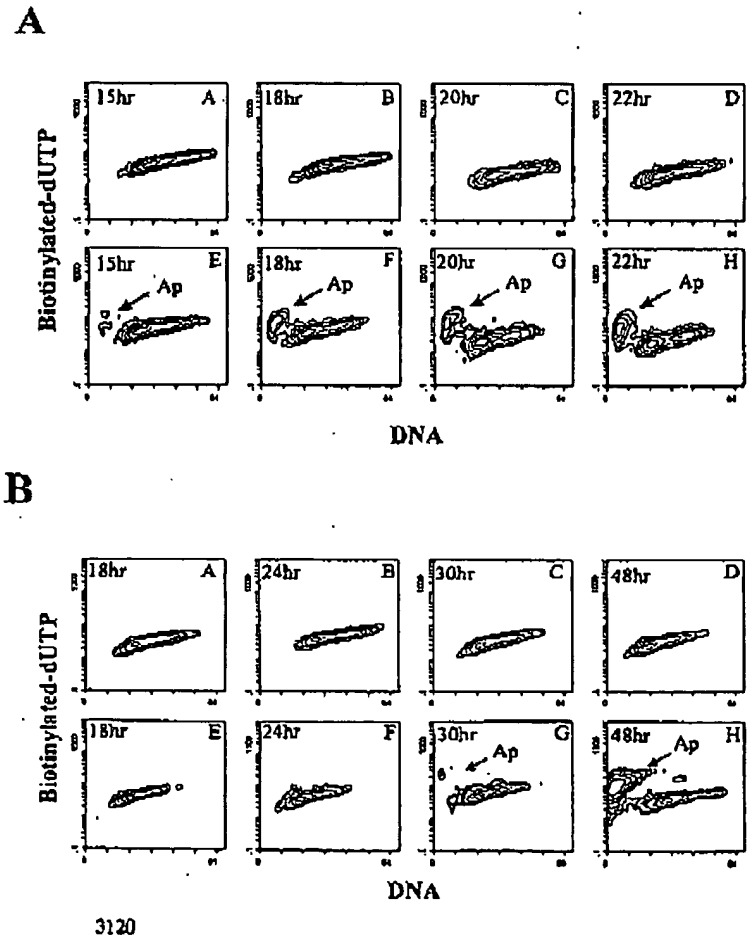

Fig. 3. Labeling of DNA breaks in apoptotic cells with biotinylated dUTP by TUNEL method. After infection, flow cytometric analysis for apoptosis was performed in a time course experiment. *A*, Tu-138 cells which are infected with dl312, a replication-defective adenovirus (*A–D*), or Ad5CMV-*p53* (*E–H*). *B*, MDA 686LN cells which are infected with dl312, a replication-defective adenovirus (*A–D*), or with Ad5CMV-*p53* (*E–H*). *Ap*, apoptosis.

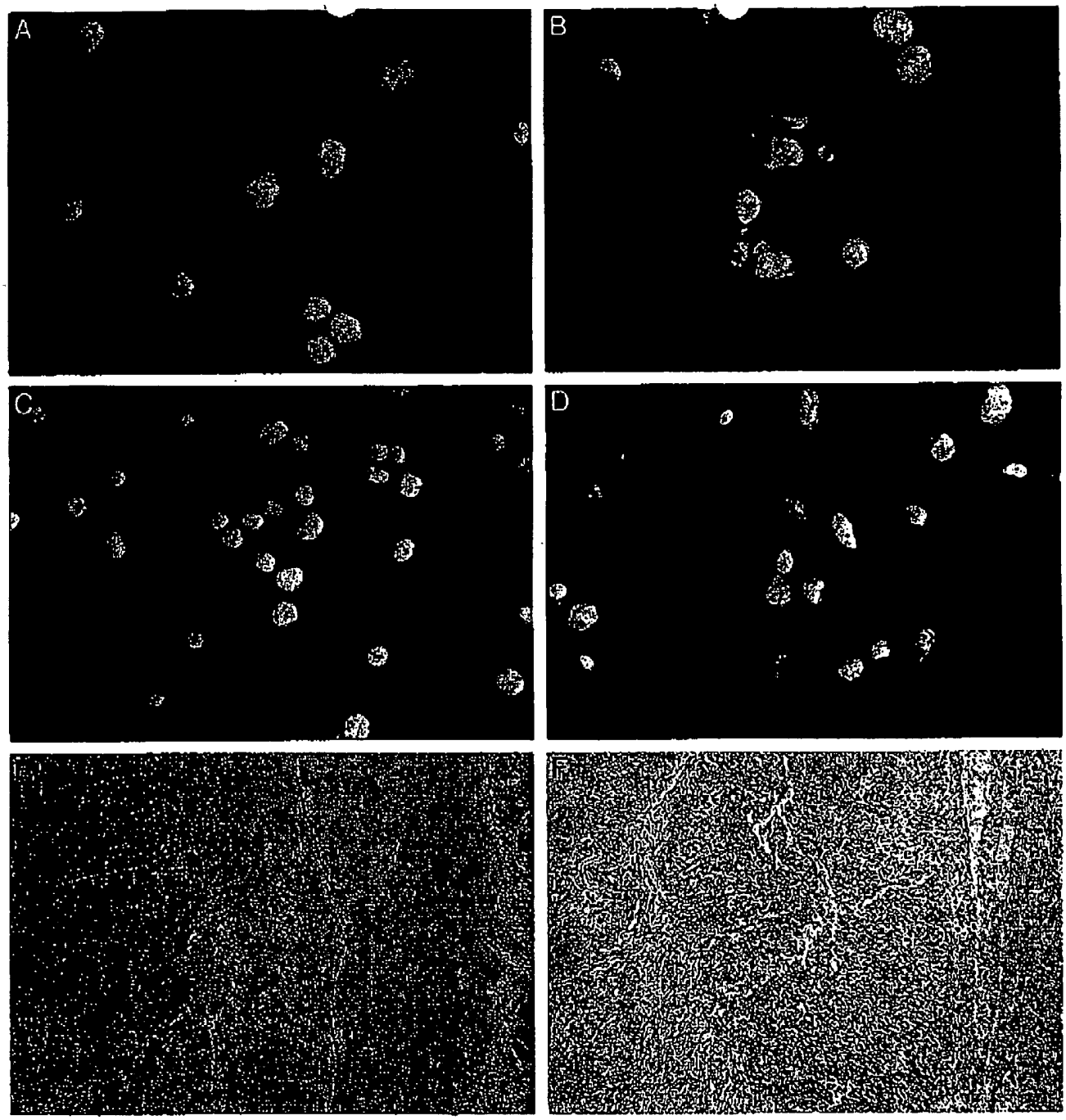

Fig. 4. *A–D*, *In vitro* analysis for apoptosis by nuclear fluorescence staining of SCCHN cell lines Tu-138 cells (*A*, *B*) and MDA 686LN cells (*C*, *D*). Twenty-two h after infection, cells were cytospun and fixed in 1% formaldehyde; the TUNEL reaction was performed as described in "Materials and Methods." Cells were then counterstained with propidium iodide to visualize their total DNA content. *A* and *C*, orange staining of both cell lines infected with replication-defective virus dl312 (100 multiplicity of infection) as a control. *B* and *D*, mixture of green (indicative of apoptosis) and orange fluorescence in both cell lines infected with Ad5CMV-*p53* adenovirus (100 multiplicity of infection). Nuclear fragmentation, characteristic of apoptosis, was only seen in the cells infected with Ad5CMV-*p53*. ×400. *E* and *F*, *In vivo* analysis for apoptosis in an animal model of microscopic residual disease. *In situ* end-labeling analysis was performed on paraffin-embedded sections obtained from MDA 686LN tumor-bearing nude mice from our previous study (13). One of the tumors was treated with only PBS as a control (*E*) and revealed no significant staining for apoptosis. The other tumor was treated with 10^7 plaque-forming units of Ad5CMV-*p53*, which suppressed tumor growth and induced extensive apoptosis within the tumor (*F*). Apoptosis was not visualized in any surrounding normal tissues. ×63.

study, despite the induction of WAF1/CIP1 protein expression in the Ad5CMV-*p53*-infected cells,[4] cell cycle analysis revealed no evidence of arrest at G_1 before apoptosis, indicating that growth arrest is not a prerequisite for apoptosis but is a distinct process. Recently, E2F-1, a transcription factor that forms a complex with the retinoblastoma susceptibility gene product, *RB*, has been shown to cooperate with *p53* in mediating apoptosis (27), indicating that the two tumor suppressor gene products *RB* and *p53* interact with each other as checkpoint control regulators of the cell cycle. The molecular mechanisms that mediate such communication, as well as cross-talk among

other important cell cycle-regulating components such as *p16*, *p21*, cyclins, and cyclin-dependent kinases remain to be elucidated.

In summary, the studies presented here demonstrated that Ad5CMV-*p53* induced apoptosis in SCCHN but spared normal cells. In contrast, Roth *et al.* (19) found that the same intervention suppressed the growth of malignant non-small cell lung cancer cells (H358) but did not induce apoptosis. This supports the concept that there are inherent constitutive differences between these neoplasms that may regulate the apoptotic process. We conclude that selective induction of apoptosis of solid malignancies that spares normal cells is an attractive strategy for molecular therapy and requires further investigation.

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